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REMARKS

Reconsideration of this Application is respectfully requested.

Upon entry of the foregoing amendments, claims 29 and 31-33 are pending in the application, with 29 being the independent claim. These changes are believed to introduce no new matter, and their entry is respectfully requested.

Based upon the above Amendments and the following Remarks, the applicants respectfully request the Examiner reconsider all outstanding objections and rejections, and that they be withdrawn.

Drawings

The Examiner states that new corrected drawings are required because figures 8 and 10 are considerably out of the center of the pages. On June 10, 2002 in response to a Notice of Filing Missing Parts mailed on April 1, 2002, substitute drawings were filed complying with the rules on drawings set forth in the Office communication. New figures 1 through 11 (13 pages) were submitted correcting the figures the Examiner cites to in her Office action. A copy of the drawings previously submitted is herewith attached. Therefore, applicants respectfully request the Examiner withdraw the objection.

Rejections under 35 U.S.C. § 101 and 35 U.S.C. § 112, 1st paragraph

Claims 29, 31, and 32 stand rejected under 35 U.S.C. §101 because the Examiner alleges the claimed invention is not supported by either a credible asserted or a well-established utility. The Examiner alleges that neither the specification as filed nor any art

of record suggest that human homozygous pluripotent stem cells are obtainable by the claimed method so that a practical utility could be well established. The Examiner further alleges that numerous pre- and post-filing publications indicate that considerable research is needed before human pluripotent stem cells could become a practical therapeutic regimen.

Claims 29, 31, and 32 also stand rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the enablement requirement because the claimed invention is not supported by either a credible asserted or a well established utility. The Examiner alleges that one skilled in the art would not know how to use the claimed invention without undue experimentation. The Examiner cites a post-filing publication by the applicants (Stem Cells 2003, 21:152-61) and argues that only mouse pluripotent stem cells were obtained. More specifically, the Examiner cites the sentence "so far, further culturing of the [human] inner cell masses produced by this activation method has not been successful."

Applicants respectfully request reconsideration and withdrawal of these rejections in view of the following remarks. Applicants respectfully believe that the Examiner has taken the cited sentence out of context. The sentence in the publication refers to one of three activation techniques employed to investigate the feasibility of using particular reagents to activate human MII oocytes to produce a viable inner cell mass for stem cell derivation. The sentence cited by the Examiner applies to the first technique. The paragraph continues on to describe two other techniques and the success in activating oocytes, which are detailed within the present specification. *See* Specification, Example 1(d), p. 45 and p. 22.

The Examiner also alleges that numerous publications regarding stem cells generated by parthenogenic oocyte activation further support that the specification fails to teach how to overcome the difficulties in the art. Based upon the specification and the techniques employed in the examples, one of ordinary skill in the art would be able to practice the invention without undue experimentation. The techniques taught in the specification provide detailed information on how to overcome the difficulties in the art. This is further supported by the applicants' reference cited by the Examiner (Stem Cells 2003, 21:152-61), which illustrates the success of the techniques described in the instant specification.

Applicants respectfully request reconsideration of this rejection and withdrawal of these grounds of rejection in view of the aforementioned remarks. Applicant submits that the pending claims satisfy the utility and enablement requirements.

Rejection under 35 U.S.C. § 112, 1st paragraph

The Examiner also asserts that it would require undue experimentation to practice the invention as it is broadly claimed to include mitotically activated sperm cells and that based upon the specification and a post-filing publication (Lin, *et al.*) it appears only female HS cells could be obtained.

Applicants respectfully request reconsideration of this rejection and withdrawal of these grounds of rejection in view of the aforementioned amendments. Applicant submits that the pending claims, as amended herein, satisfy the enablement requirement.

Rejection under 35 U.S.C. § 112, 1st paragraph

The Examiner also asserts that the specification fails to teach whether serological techniques are suitable for HS cell typing since these cells are pluripotent, and yet to develop mature HLA serotypes. Applicants respectfully request reconsideration of this rejection and withdrawal of this ground of rejection in view of the following remarks.

According to Example 2 in the specification, "[o]nce HS cell populations have developed . . . a sample of each population can be subjected to in vitro differentiation for the optimal expression of HLA molecule, the HS-derived sample cells from each population are then tested for HLA-A, -B, and –C specificities using the microlymphotoxicity assay." Specification, p. 51; *see* also p. 5, Example 4. The specification provides a detailed explanation of serological techniques that can be employed for HS cell typing. *See* Specification, p. 24-26.

Applicants respectfully request reconsideration of this rejection and withdrawal of these grounds of rejection in view of the aforementioned remarks. Applicant submits that the pending claims satisfy the enablement requirement.

Rejection under 35 U.S.C. § 112, 2nd paragraph

Claims 29 and 31-33 stand rejected under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. The Examiner asserts that claims 29 and 31-33 are vague and indefinite because of the claim recitation, "post-meiosis I diploid germ cells". Given the plain meaning of the phrase, the Examiner believes the term refers to germ cells in the meiotic division phase II as being haploid rather than diploid. Further, the examiner alleges that it is unclear what stage the germ cells are encompassed by the claims and thus the metes and bounds of the claims are uncertain.

Applicants respectfully traverse this rejection. On page 17 of the instant specification, the term "homozygous post-meiosis I diploid germ cells" is defined as "germ cells that are the stage of gametogenesis at which the cells contain two copies of either the paternal or maternal homologous chromosomes." It is well known in the art that prior to the first meiotic division, the chromosomes of the cell (2n), undergo replication giving rise to a 4n cell. After the first meiotic division, the number of

chromosomes is reduced by half, resulting in two 2n cells, each of which has 46 chromatids (diploid). *See* Figure 8-2, Lodish, *et al.*, Molecular Cell Biology, 2000, attached as Exhibit 1. If the second meiotic division were to occur it would result in four 1n (haploid) cells as a result of the cells dividing again without intervening DNA replication. Further, the specification states that "[h]aploid oocytes resulting from activation are able to self-replicate their genome without cytokinesis and give rise to diploid cells." *See* Specification, p. 45, Example 2; *see* also Taylor, A.S., *et al.*, Hum. Reprod. 9(12):2389-97 (1994) and Kaufman, M.H., *et al.*, J. Embryol. Exp. Morphol. 73:249-61 (1983), attached as Exhibit 2 and 3, respectively. Therefore, one of ordinary skill in the art would understand what stage the germ cells are at in the claims.

Applicants respectfully request reconsideration of this rejection and withdrawal of these grounds of rejection in view of the aforementioned remarks. Applicant submits that the pending claims particularly point out and distinctly claim the invention.

Rejection under 35 U.S.C. § 112, 2nd paragraph

The Examiner also alleges that claim 29 is vague and indefinite because it is unclear what characteristics the term "homozygous" defines in the term "homozygous stem cells". The Examiner asserts that the metes and bounds of the claim are unclear because it is unknown whether "homozygous" is defined as the size, type, or MHC homozygosity and genomic loci of the stem cells.

Applicants respectfully traverse this rejection. According to the specification on page 8 and throughout the application, the focus of the invention is on the Major Histocompatibility Complex (MHC). The specification also states that "a cell is classified as 'homozygous' if it contains two copies of the same allele." Specification, p. 17. It is also important to point out that the activated haploid replicates after the stage where potential for crossing over occurs (during meiosis I), which gives rise to identical copies resulting in homozygous stem cells. Therefore, the stem cells are homozygous

with respect to MHC, which pertains to the "immunotyped homozygous stem cells" in the claims.

Applicants respectfully request reconsideration of this rejection and withdrawal of these grounds of rejection in view of the aforementioned remarks. Applicant submits that the pending claims are definite.

Rejection under 35 U.S.C. § 112, 2nd paragraph

The Examiner also alleges that claim 29 is vague and indefinite because it is unclear which type of stem cells are encompassed in the claim by the term "stem cells". The Examiner further notes that the specification teaches that the claimed HS cells are pluripotent (Specification, page 6, last paragraph), yet the claims encompass EM stem cells or tissue specific progenitor cells.

The applicants respectfully traverse this rejection. According to the specification on pages 14-15, "stem cell" is defined as, "a relatively undifferentiated cell that actively divides and cycles, giving rise upon proper stimulation to a lineage of mature, differentiated, functional cells. The defining properties of a stem cell include: (a) it is not itself terminally differentiated; (b) it can divide without limit for the lifetime of the animal; and (c) when it divides, each daughter has a choice of remaining a stem cell or embarking on a course that leads irreversibly to terminal differentiation. Those stem cells that are initially unrestricted in their capabilities (i.e., capable of giving rise to several types of differentiated cell) are called 'pluripotent'. Current sources of pluripotent cells include embryonic (ES) stem cells, embryonic carcinoma (EC) cells, cells generated from somatic cloning, teratomas, and teratocarcinomas." Therefore, since the term is defined in the specification to mean "pluripotent," the claims pertain to "pluripotent" stem cells.

Applicant respectfully requests reconsideration of this rejection and withdrawal of these grounds of rejection in view of the aforementioned remarks.

CONCLUSION

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. It is believed that a full and complete response has been made to the outstanding Office action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Response is respectfully requested.

Respectfully submitted,

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EXHIBITS

Exhibit 1: Genetic Analysis in Cell Biology.

Exhibit 2: Kaufman, M.H., et al., J. Embryol. Exp. Morph., 73:249-261 (1983).

Exhibit 3: Taylor, A.S., et al., Human Reproduction, 9(12):2389-2397 (1994).

FOURTH EDITION

MOLECULAR CELL BIOLOGY

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Arnold Berk
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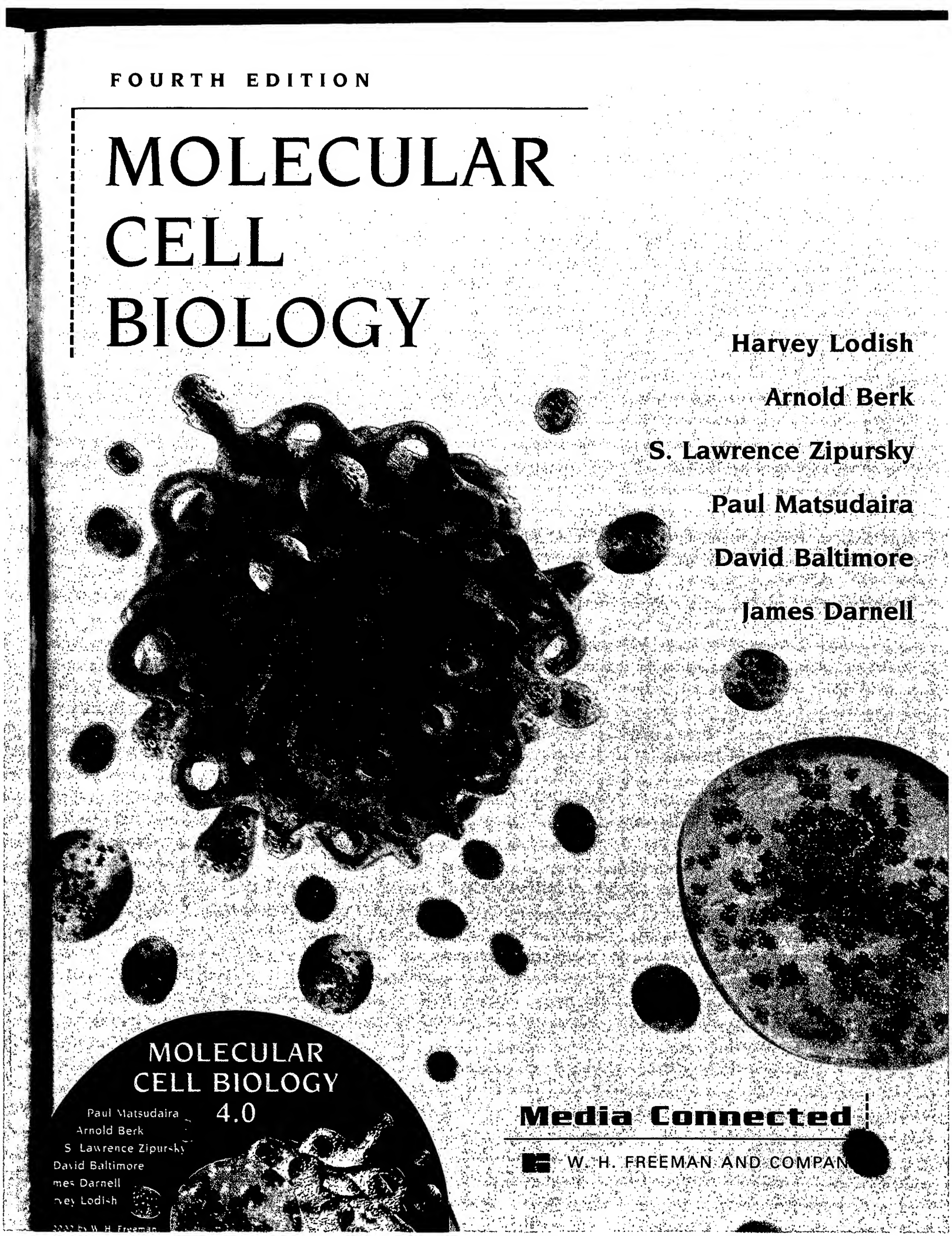

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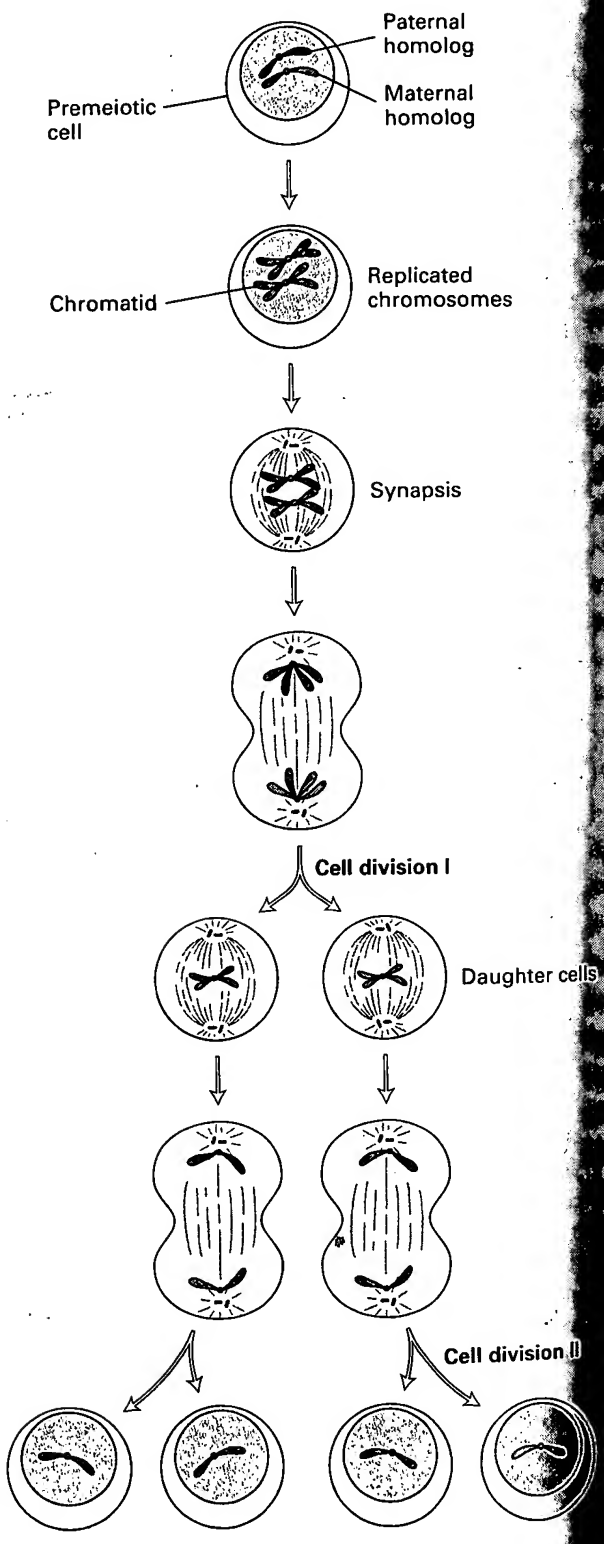
as *haplo-insufficient*. In other cases, mutations in one allele may lead to a structural change in the protein that interferes with the function of the wild-type protein encoded by the other allele. These are referred to as *dominant negative mutations*.

Some alleles can be associated with both a recessive and a dominant phenotype. For instance, fruit flies heterozygous for the mutant *Stubble (Sb)* allele have short and stubby body hairs rather than the normal long, slender hairs; the mutant allele is dominant in this case. In contrast, flies homozygous for this allele die during development. Thus the recessive phenotype associated with this allele is lethal, whereas the dominant phenotype is not.

Inheritance Patterns of Recessive and Dominant Mutations Differ

Recessive and dominant mutations can be distinguished because they exhibit different patterns of inheritance. To understand why, we need to review the type of cell division that gives rise to **gametes** (sperm and egg cells in higher plants and animals). The body (somatic) cells of most multicellular organisms divide by mitosis (see Figure 1-10), whereas the **germ cells** that give rise to gametes undergo **meiosis**. Like body cells, premeiotic germ cells are diploid, containing two of each morphologic type of chromosome. Because the two members of each such pair of **homologous chromosomes** are descended from different parents, their genes are similar but not usually identical. Single-celled organisms (e.g., the yeast *S. cerevisiae*) that are diploid at some phase of their life cycle also undergo meiosis (see Figure 10-54).

Figure 8-2 depicts the major events in meiosis. *One* round of DNA replication, which makes the cell $4n$, is followed by *two* separate cell divisions, yielding four haploid ($1n$) cells that contain only one chromosome of each homologous pair. The apportionment, or **segregation**, of homologous chromosomes to daughter cells during the first meiotic division is random; that is, the maternally and paternally derived members of each pair, called homologs, segregate independently, yielding germ cells with different mixes of paternal and maternal chromosomes. Thus parental characteristics are reassorted randomly into each new germ cell during meiosis. The number of possible varieties of meiotic segregants is 2^n.



▶ **FIGURE 8-2 Meiosis.** A premeiotic germ cell has two copies of each chromosome ($2n$), one maternal and one paternal. Chromosomes are replicated during the S phase, giving a $4n$ chromosomal complement. During the first meiotic division, each replicated chromosome (actually two sister chromatids) aligns at the cell equator, paired with its homologous partner; this pairing off, referred to as *synapsis*, permits genetic recombination (discussed later). One homolog (both sister chromatids) of each morphologic type goes into one daughter cell, and the other homolog goes into the other cell. The resulting $2n$ cells undergo a second division without intervening DNA replication. During this second meiotic division, the sister chromatids of each morphologic type separate and these now independent chromosomes are randomly apportioned to the daughter cells. Thus, each diploid cell that undergoes meiosis produces four haploid cells, whereas each diploid cell that undergoes mitosis produces two diploid cells (see Figure 1-10).

J. Embryol. exp. Morph. 73, 249-261, 1983

Establishment of pluripotential cell lines from haploid mouse embryos

By M. H. KAUFMAN[1], E. J. ROBERTSON[2], A. H. HANDYSIDE[2,3] AND M. J. EVANS[2]

From the Departments of Anatomy and Genetics, University of Cambridge

SUMMARY

Eggs from 129 SvE and (C57BL × CBA)F_1 hybrid female mice were activated parthenogenetically following their exposure to a 7 % solution of ethanol in PBS. Only the haploid class which developed a single pronucleus following second polar body extrusion was examined further. These eggs were transferred to suitable recipients and 'delayed' blastocysts subsequently recovered. The 'delayed' blastocysts were explanted into tissue culture and a total of four haploid-derived pluripotent cell lines established from individual embryos. Chromosome analysis of morulae revealed that over 80 % contained only haploid mitoses. However, chromosome analysis of early passage cell lines revealed that all were diploid with a modal number of 40 chromosomes. When transplanted into syngeneic hosts, all lines formed well-differentiated teratocarcinomas. This technique provides a source of homozygous diploid cell lines of parthenogenetic origin.

INTRODUCTION

It has been suggested that the direct isolation of pluripotential cells from early embryos might provide sources of pluripotential cells with a karyotype unchanged from that of the embryo from which it was derived (Evans, 1981). In addition to their relative ease of production from blastocysts, the principal advantage that such EK cell lines have over most currently available embryonal carcinoma (EC) cell lines derived from tumours is that, at least initially, they do have a normal karyotype (Evans & Kaufman, 1981).

Recent studies in which pluripotential cell lines have been established from a considerable number of individual fertilized mouse embryos with both a normal and abnormal chromosome complement (Martin, 1981; Evans, Robertson, Bradley, Handyside & Kaufman, unpublished) have stimulated us to attempt to

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establish similar pluripotential cell lines from haploid parthenogenetically-derived embryos.

MATERIALS AND METHODS

i. *Ethanol activation*

Eight- to 12-week-old 129 SvE and (C57BL×CBA)F_1 hybrid female mice were superovulated with 5 i.u. PMSG followed 48 h later by 5 i.u. HCG. The females were autopsied 17 h later and the cumulus masses recovered from the oviducts released into a freshly prepared 7% (v/v) solution of Analar quality ethanol in Phosphate Buffered Saline (PBS) containing both Ca^{2+} and Mg^{2+}, and retained in this solution for about 4½ min. Cumulus masses from four to six females were pooled together, treated as a single group, and this and the following washing procedures were carried out at room temperature. The cumulus masses were washed through three changes of ethanol-free PBS and through two changes of embryo culture medium (Whittingham, 1971). Individual cumulus masses were then transferred to separate drops of medium under paraffin oil and incubated for 4–5 h at 37 °C in an atmosphere of 5% CO_2 in air. The adherent cumulus cells were then removed with hyaluronidase, and the overall activation frequency determined and the various classes of parthenogenone induced separated into different groups (Kaufman, 1978*a*). Only those activated oocytes that developed a single haploid pronucleus following second polar body extrusion were used in this study. A more detailed description of this ethanol activation technique has been published elsewhere (Cuthbertson, Whittingham & Cobbold, 1981; Kaufman, 1982).

A proportion of the pronucleate-stage 1-pronuclear haploid eggs were transferred to the oviducts of recipients (Tarkowski, 1959) anaesthetized with Avertin on the afternoon of the first day of pseudopregnancy (i.e. on the day in which the vaginal plug had earlier been observed, following mating of the female with a vasectomized male), while others were retained in culture.

The recipients were divided into two groups. The first group was ovariectomized on the afternoon of the 4th day of pseudopregnancy and, while they were still under the influence of the anaesthetic, given a subcutaneous injection of 1 mg Depo-Provera (Upjohn). This group of females was subsequently autopsied 4–5 days later, the uterine horns removed and flushed with PBS in order to recover delayed blastocysts. The second group of recipients was autopsied at about midday on the 4th day of pseudopregnancy and the reproductive tract flushed with PBS. About half of the recovered embryos, which were mostly at the morula stage, were then incubated for about 3 h in medium containing 1 μg/ml Colcemid. These embryos were then examined by the air-drying technique (Tarkowski, 1966), and the preparations stained with Giemsa. It was possible to classify almost all of the embryos with cells in division into three distinct groups, namely i. haploid, ii. haploid-diploid mosaics or iii.

diploid, according to the number of chromosomes present in the individual metaphase plates. The embryos from this group of recipients that were not examined by air drying were allowed to develop to the blastocyst stage in culture, then transferred to the uterine horns of other recipients in order to recover delayed blastocysts.

The embryos that were retained in culture from the 1-cell stage were subsequently transferred, at about 74–75 h after activation, into medium containing 1 μg/ml Colcemid for 3–4 h, and air-dried preparations made as described above. The ploidy of this group of embryos was also determined.

ii. *Establishment of pluripotent cell lines from delayed blastocysts*

Individual delayed blastocysts, many of which contained large clearly delineated inner cell masses (ICMs) (Fig. 1A) were transferred to tissue culture

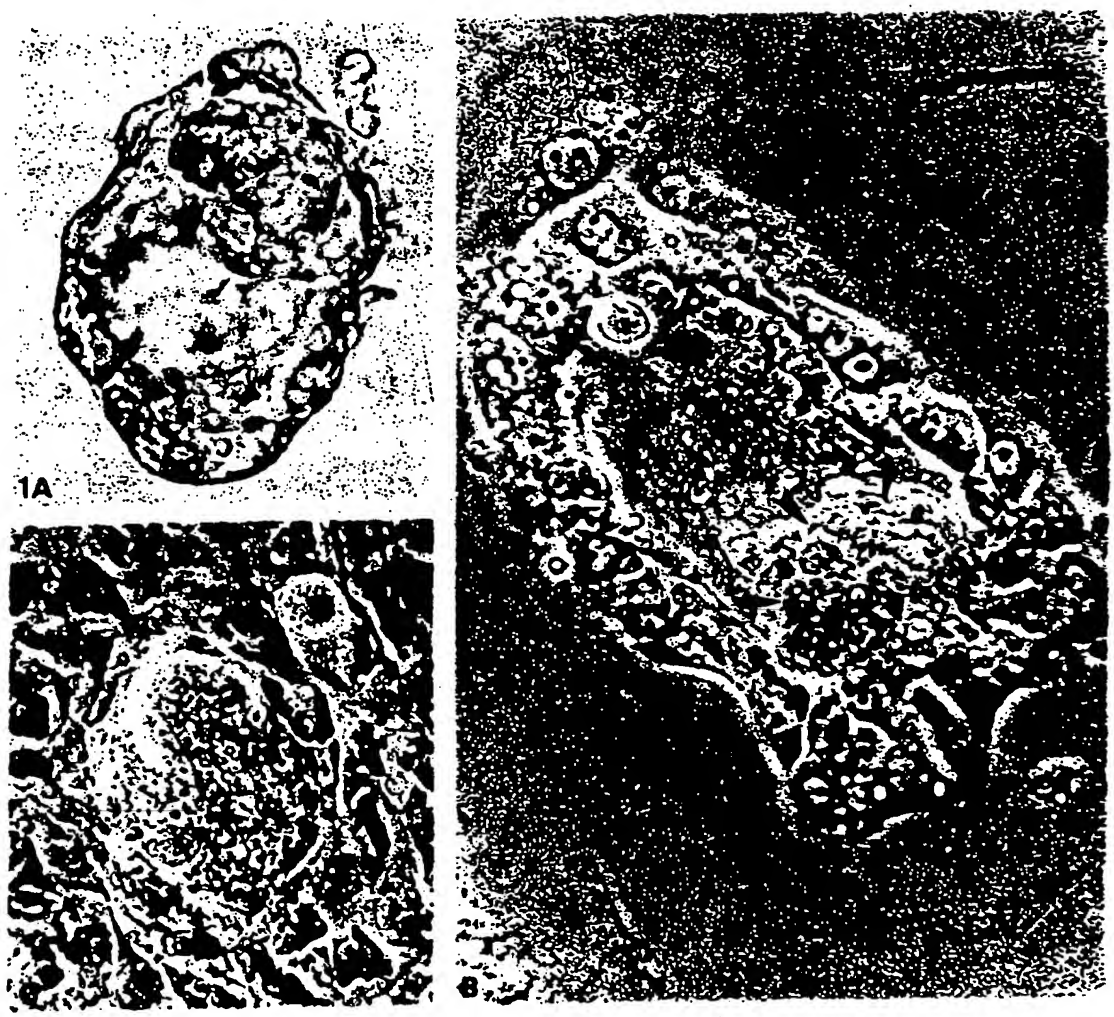


Fig. 1. A. 'Delayed' 129 SvE blastocyst shortly after its explantation into tissue culture medium. Note the large inner cell mass. B. Appearance of 'implanted' blastocyst at approximately 60 h after explantation. Note centrally-located clump of inner-cell-mass-derived cells (arrow). C. A group of haploid-derived cells, growing on a feeder layer, shortly after their establishment in culture.

dishes containing Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% foetal calf serum and 10% newborn calf serum (Evans & Kaufman, 1981). After blastocyst attachment, which usually occurred within 48 h of explantation (Fig. 1B), the ICM-derived cell clumps were selectively removed following an additional 4 days of culture. The ICM clumps were disaggregated in 0·25% (w/v) trypsin, 0·04% (w/v) EDTA and replated onto feeder layers of mitomycin-treated fibroblasts. In successful cultures nests of stem cells appeared following two rounds of cell growth and trypsinization. These cells have a distinctive morphology in culture (Fig. 1C) closely resembling other established tumour-derived and embryo-derived pluripotential cell lines. The haploid-derived (HD) lines were subsequently maintained on feeder layers and subcultured at 4–6 day intervals.

iii. *Testing of differentiation ability of the cell lines*

The differentiation ability of the 129 SvE lines was tested by inducing tumour formation in syngeneic host animals. For each line 10–12 male 129 SvE mice were inoculated subcutaneously with approximately 10^6 cells. Tumour masses were retrieved after 4 to 6 weeks and fixed in Bouin's solution, dehydrated and subsequently serially sectioned at a nominal thickness of 7 μm. Alternate slides were then either stained with haematoxylin and eosin or with Masson's trichrome.

iv. *Chromosome analysis of cell lines*

Chromosomal analysis was performed on early passage cell lines. This was usually carried out within five to ten passages following the original disaggregation of the ICM-derived cell clumps. The chromosomes were analysed by G-banding (modification of the A.S.G. procedure of Gallimore & Richardson, 1973), and karyograms arranged according to the nomenclature of Nesbitt & Francke (1973).

RESULTS

A. *Observations on the activation rate and incidence of the various classes of parthenogenone induced*

Observations on the incidence of the various classes of parthenogenone induced when 129 SvE and (C57BL × CBA)F_1 hybrid oocytes isolated at 17 h after the HCG injection for superovulation were stimulated by exposure to a 7% solution of ethanol in PBS for about 4½ min are presented in diagrammatic form in Fig. 2. The data included in this figure are the combined results of all the activation studies carried out over a period of several months involving these two strains of mice, and in all represent the results of isolated experiments carried out on more than 10 separate occasions.

In both strains, the highest proportion of the activated population consisted of haploid parthenogenones which had developed a single (haploid) pronucleus

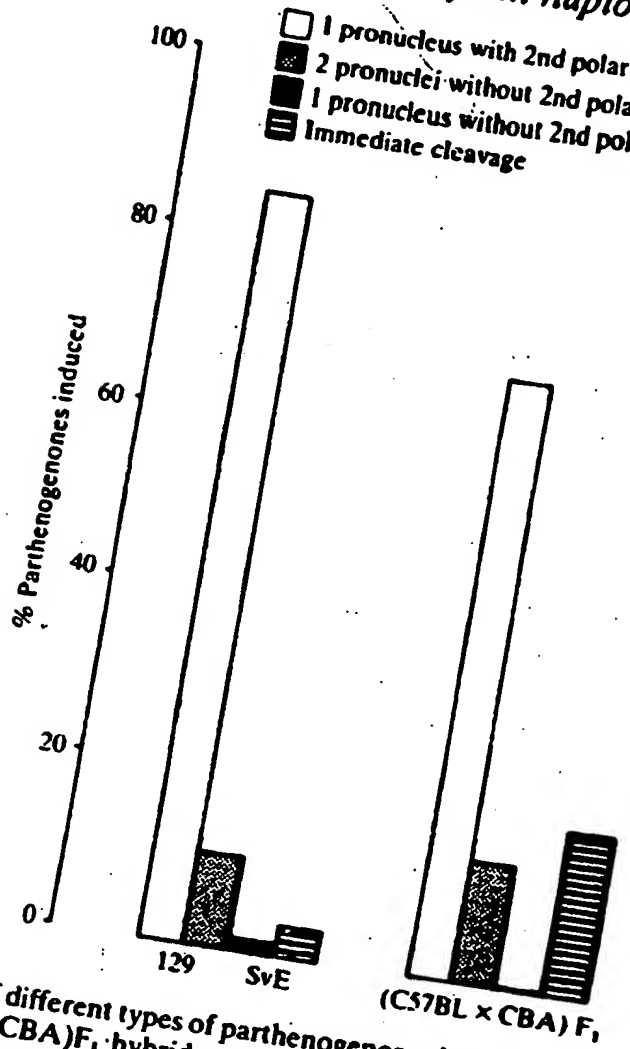


Fig. 2. Incidence of different types of parthenogenones induced when eggs from 129 SvE and (C57BL × CBA)F_1 hybrid mice were briefly incubated in 7 % ethanol in PBS. Cumulus masses were released at 17 h after HCG and observations made 4–5 h later. The total number of activated eggs examined in the 129 SvE series was 1351. and the activation frequency was 80·0 %. In the F_1 series, 1211 activated eggs were examined, and the activation frequency was 95·7 %.

following extrusion of the second polar body. The overall activation rate in both strains was high, with about 80–95 % of the oocytes exposed to the ethanol treatment being stimulated to develop parthenogenetically. As indicated in the Methods section, only the 1-pronuclear haploid embryos were used subsequently in the present study.

B. *Chromosome analysis at about 75–77 h after activation*

i. In vivo *series*

Activated haploid eggs from 129 SvE and (C57BL × CBA)F_1 hybrid females which had previously been transferred to the oviducts of suitable recipients at the 1-cell stage, were isolated at about midday on the 4th day of pseudopregnancy. The embryos, which were largely at the morula stage, were then incubated for about 3 h in medium containing 1 μg/ml Colcemid, and subsequently examined

by the air-drying technique described by Tarkowski (1966). As only about half of the recovered embryos were used to assess the ploidy, the others being transferred to additional recipients in order to obtain delayed blastocysts, only details of the fixed embryos with cells in division will be presented here (see Table 1). In the haploid–diploid mosaic embryos only one or two diploid metaphases were usually present, and almost all of the mitoses observed in this group were haploid. In the 129 SvE series 82 %, and in the (C57BL × CBA)F_1 series 85 % of the embryos examined had only haploid mitoses present (see Fig. 3).

ii. In vitro *series*

In a parallel series of experiments, (C57BL × CBA)F_1 hybrid oocytes were activated *in vitro* with ethanol and the 1-pronuclear haploids retained in culture until about midday on the 4th day (about 73–74 h after activation), then those that had progressed beyond the 4-cell stage were transferred to medium containing 1 μg/ml Colcemid for 3–4 h. Out of an initial total of 174 1-cell activated eggs, 157 embryos had more than four cells present by the early afternoon on the 4th day, but by this time most of the embryos were at the morula stage of development. Air-dried preparations were made as described above. In 12 of these embryos no cells were in division, in 141 embryos one or more mitoses were present, and in 4 embryos virtually all of the cells were in division and it was considered impossible to make an assessment of the ploidy because of extensive overlapping of mitotic figures. Of the 141 embryos with cells in division, 102 (72 %) had only haploid mitoses, 35 (25 %) had both haploid and diploid mitoses present, while 4 (3 %) had only diploid mitoses present (see Table 1). The mean number of cells (±S.E.) in the haploid, haploid-diploid mosaic and diploid embryos in this series was 18·2 ± 0·6, 16·4 ± 0·9 and 14·0 ± 4·3, respectively, while the mean number of cells in mitosis in each of these groups of embryos was 5·2 ± 0·3, 6·6 ± 0·6 and 4·0 ± 1·7, respectively. Following the 3–4 h period of incubation in medium containing Colcemid, approximately 30–40 % of the blastomeres in these embryos were therefore blocked in mitosis at the time of analysis. In the haploid–diploid

Table 1. *Chromosome analysis of 1-pronuclear haploid embryos at the morula stage of development*

Group	Strain	Total embryos with mitoses	Ploidy: Haploid	Haploid-Diploid	Diploid
Oviduct transfer embryos	129 SvE	17	14 (82%)	2	1
	(C57BL × CBA)F_1	78	66 (85%)	5	7
in vitro culture	(C57BL × CBA)$_1$	141	102 (72%)	35	4

1-cell embryos were transferred to the oviducts of recipients on the afternoon of the first day and isolated at midday on the 4th day of pseudopregnancy.

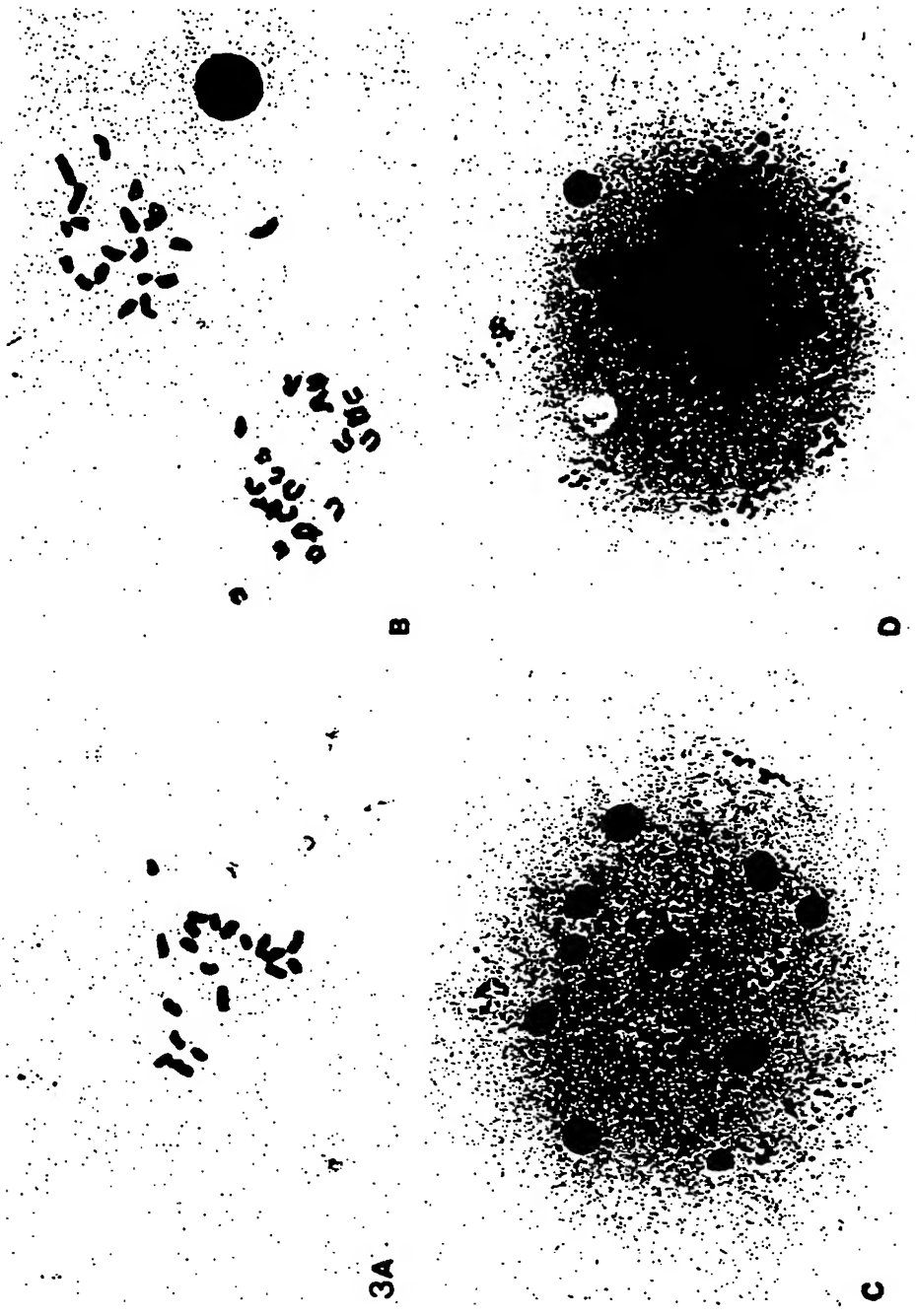


Fig. 3. Air-dried preparations stained with Giemsa. A. Haploid first cleavage metaphase spread with 20 chromosomes present. B. Two haploid metaphases from a 17-cell haploid morula. C. Morula with 7 haploid mitoses. D. Haploid–diploid morula with 10 haploid and a single diploid mitosis (arrow).

mosaics, only one or occasionally two diploid mitoses were observed, and almost all of the mitoses present were haploid.

C. *Recovery of delayed blastocysts*

Out of an initial total of 327 129 SvE and 627 (C57BL × CBA)F_1 hybrid pronucleate-stage 1-pronuclear haploid eggs transferred to the oviducts of

recipients on the afternoon of the first day of pseudopregnancy, 64 129 SvE (20%) and 104 F_1 hybrid (17%) delayed blastocysts were subsequently recovered. The delayed blastocysts were then transferred to tissue-culture medium supplemented with serum. After 72–96 h, when the majority of embryos had 'implanted', the inner-cell-mass-derived lumps were either disaggregated in an attempt to determine their ploidy (see Section F), or retained in culture to establish pluripotent cell lines (see Section D).

D. *Establishment of cell lines in culture*

Four haploid-derived cell lines have so far been established. These lines were derived on three separate occasions over a period of several months from both 129 SvE and (C57BL × CBA)F_1 hybrid delayed blastocysts (Table 2). The origin of the various lines was confirmed by GPI isozyme analysis, as the 129 SvE-derived lines were homozygous for the Gpi-1^a isozyme, and the F_1 derived lines homozygous for the Gpi-1^b isozyme of glucose phosphate isomerase.

Table 2. *Haploid-derived pluripotent cell lines*

	Strain of origin	Lines established	Modal chromosome number
1.	129 SvE	HD1	40
2.	"	HD2	40
3.	(C57BL × CBA)F_1	HD3	40
4.	"	HD4	40

E. *Differentiation ability of pluripotent cell lines*

Both the HD1 and HD2 cells formed typical well-differentiated teratocarcinomas when injected subcutaneously into syngeneic hosts. A wide range of easily recognizable cell types were present (Fig. 4A–F), in addition to nests of undifferentiated embryonal carcinoma cells. *In vitro*, all four lines formed typical simple and cystic embryoid bodies following suspension culture of cell aggregates. Cells from lines HD3 and HD4 have recently been injected into syngeneic hosts, but the results have yet to be analysed.

F. *Chromosome analysis of pluripotent cell lines*

Repeated attempts to determine the chromosome constitution and ploidy of the ICM-derived clumps between 72 and 96 h after blastocyst explantation have so far been unsuccessful. Despite prolonged culture in Colcemid (6–12 h), no cells have been observed in division. Parallel observations on fertilized material at similar stages of development have also failed to demonstrate cells in division (authors, unpublished observations). This appears to be a technical problem, either because the cells are not in division at the time of analysis, or because of problems associated with the disaggregation of the small clumps of cells which

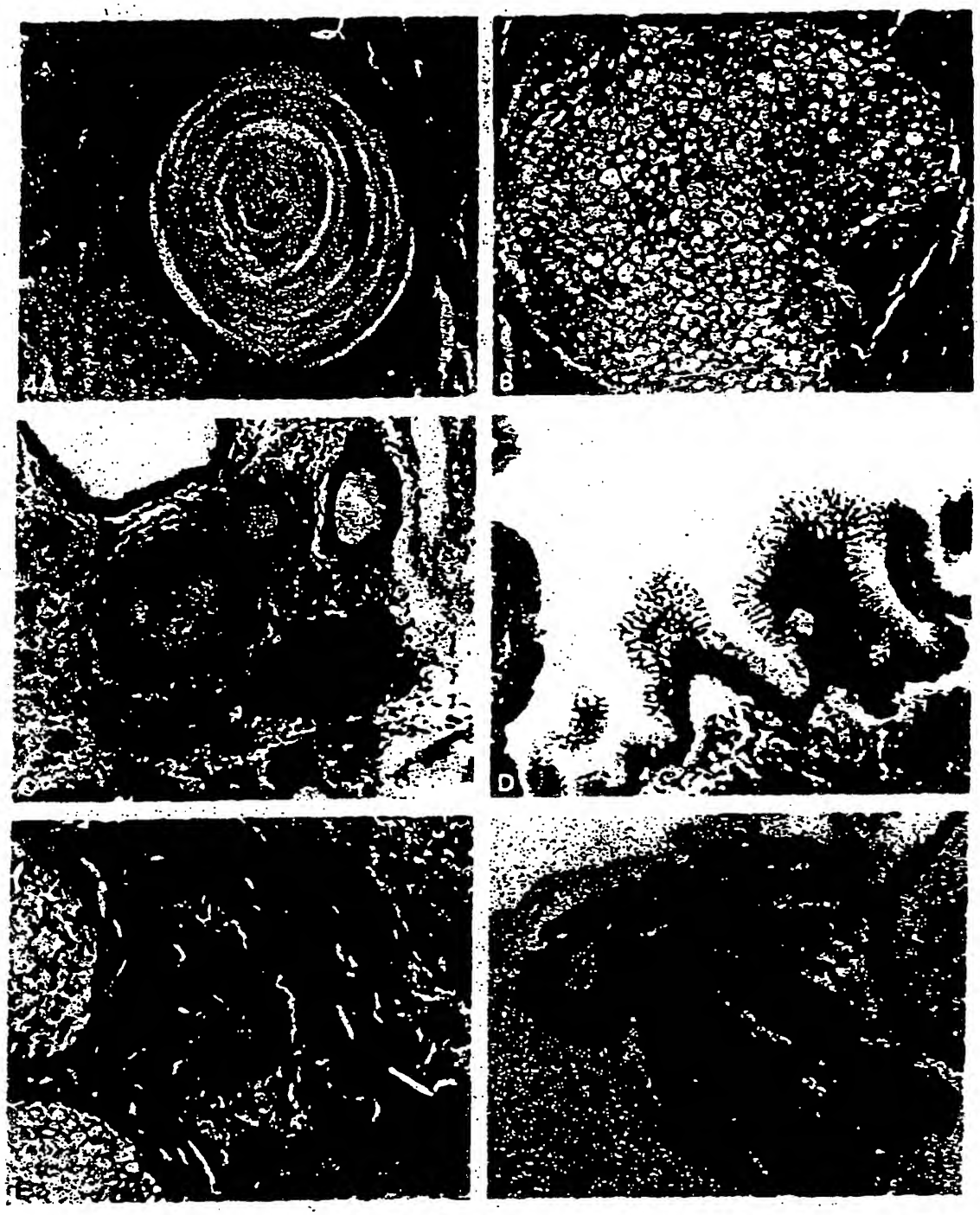


Fig. 4. Representative regions through a well-differentiated 129 SvE haploid-derived teratocarcinoma. Sections stained with Masson's trichrome. A. Keratin whorl. B. Cartilage nodule. C. Region showing areas of melanin pigmentation, precartilage nodule and epithelial-lined tubules. D. Detail from wall of large cyst lined by secretory epithelial cells. E. Area showing tissue interspersed with yolk-sac-like material. F. Organized structure formed from folded layers of columnar epithelial cells.

were tightly adherent and failed to separate following standard disaggregation techniques.

Chromosome analysis of early passage cultures revealed that all cells observed

in division at this stage were diploid – no haploid cells were detected. All four cell lines proved to have a modal number of 40, as expected. G-banding studies of 30–35 metaphase spreads from each of the lines examined confirmed that all the cell lines had a normal diploid autosomal complement. Interestingly, all the lines at the time of karyotyping, were characterized by the possession of a deletion of the distal end of the X chromosome. However, in the HD4 line, of the 31 banded spreads which were karyotyped shortly after its establishment in culture, this abnormality was only present in 16 of the metaphase spreads.

Karyograms from the HD4 line are presented in Fig. 5. In Fig. 5A, a normal karyogram is observed, whereas in Fig. 5B the karyogram showing a deletion of approximately 25 % of the distal part of a single X chromosome is presented.

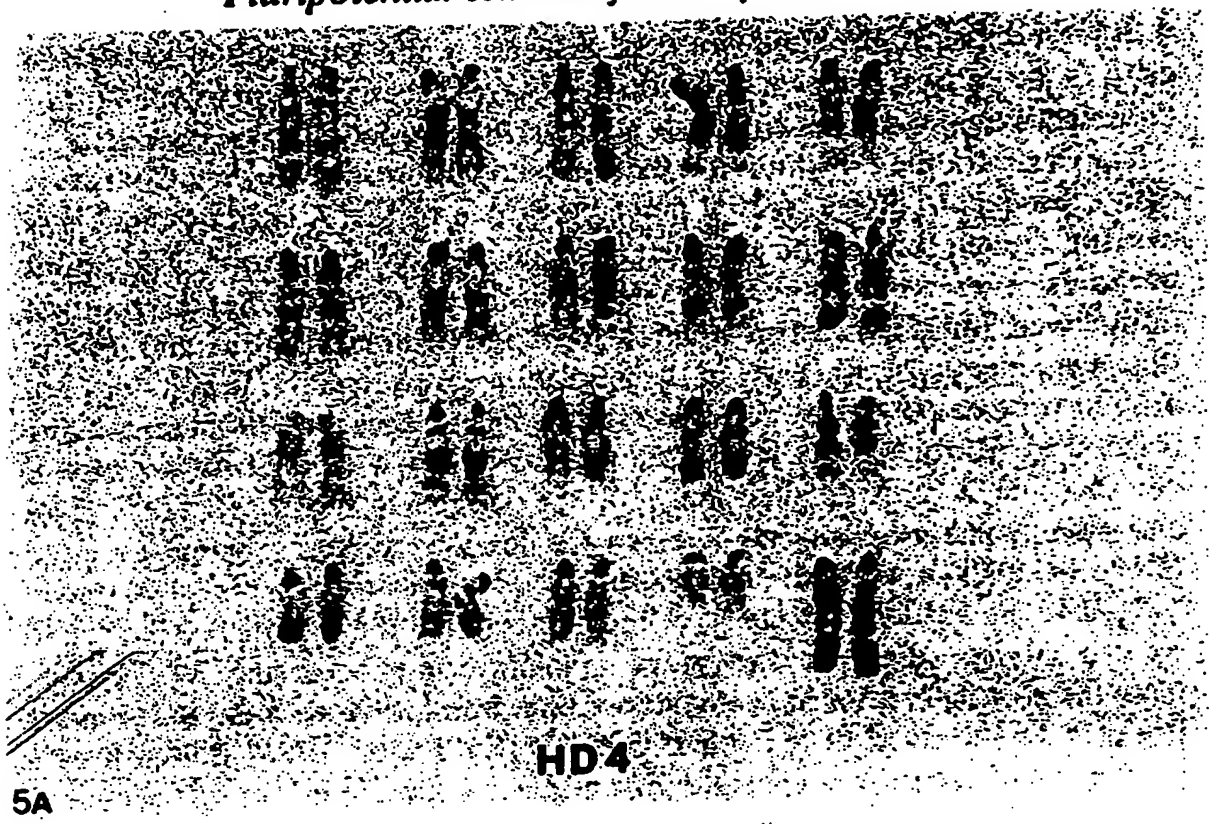
DISCUSSION

We have demonstrated that it is technically feasible to establish pluripotential cell lines from haploid embryos. These cells which were derived from haploid parthenogenones from various strains have all the properties expected in that once established in culture, they can be induced to differentiate both *in vivo* into typical teratocarcinomas with a wide variety of cell types present, and *in vitro*.

Previous attempts to establish haploid teratocarcinomas and to derive cell lines from these sources were only partially successful in that while tumours were derived from the ectopic transfer of haploid parthenogenones (Iles *et al.*, 1975; Graham, McBurney & Iles, 1975), no permanent pluripotential lines have been reported. Lines have, however, been established from spontaneous teratocarcinomas occurring in the ovaries and testes of LT/Sv strain mice, but these are undoubtedly diploid (Martin *et al.*, 1978) and some lines appear to be restricted in their differentiation (Gachelin, cited in Nicholas *et al.*, 1976). However, in the only published report in which LT-derived teratocarcinoma cells were injected into blastocysts, Illmensee (1978) reported that in one instance out of eight chimaeric individuals obtained, the tumour-derived cells not only took part in normal tissue differentiation but even contributed to the germ line.

Chromosomal analysis carried out at different stages in the establishment of the lines reported in this paper indicated that 15–18 % of the embryonic population at the morula stage contained at least a proportion of diploid cells. In the *in vivo* series no significant difference was observed between the 129 SvE- and F_1-derived embryos in the numbers of haploid vs. haploid–diploid and diploid mitoses. A difference is apparent, however, between the *in vivo* and *in vitro* series in this regard, since more diploid mitoses were seen in the latter group (see Table 1). This may be a reflection of the fact that conditions *in vitro* may be suboptimal compared to those *in vivo* for the maintenance of haploidy during the early preimplantation period.

Several attempts to determine the chromosome constitution of the delayed blastocysts within 3–4 days after their isolation and explantation into culture



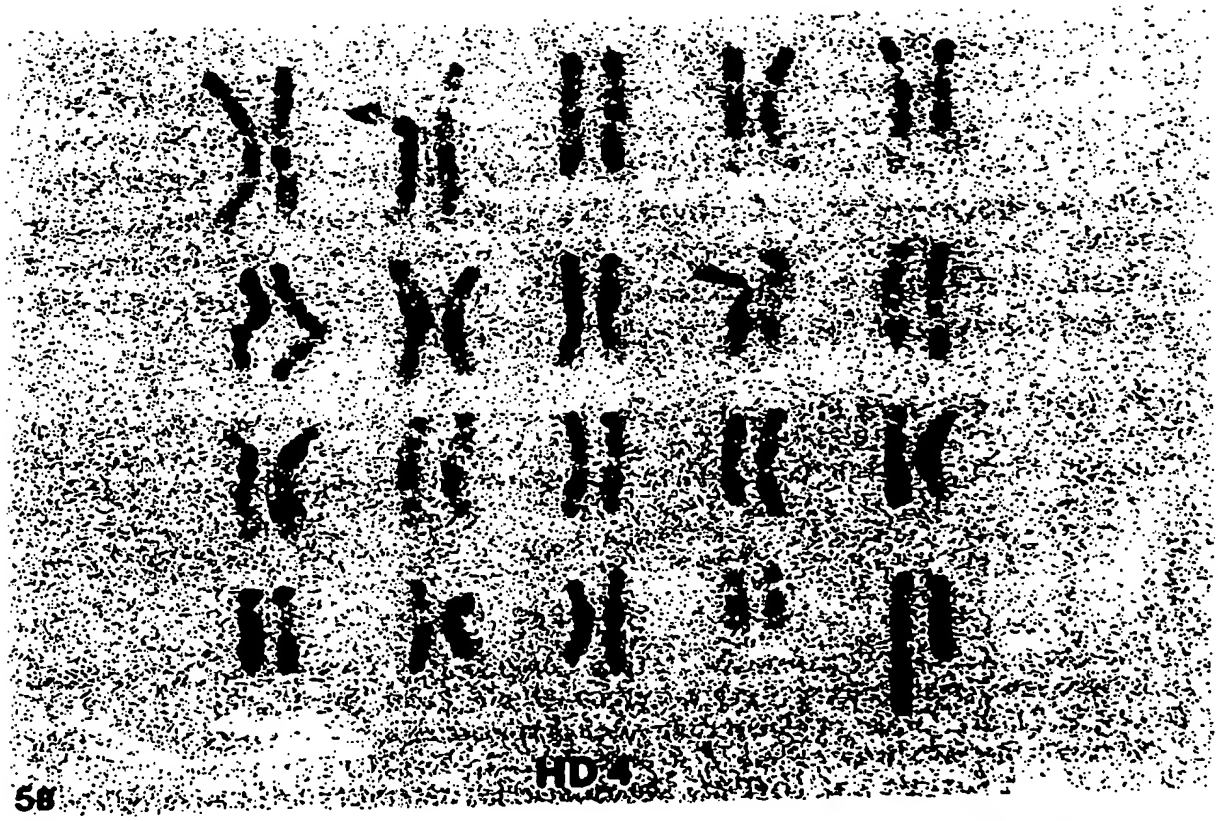


Fig. 5. Karyograms from HD4 line. A. Showing normal XX euploid chromosome complement. B. Showing deletion of approximately 25 % of the distal region of one of the X chromosomes.

were unsuccessful, as no mitoses were observed in the inner-cell-mass-derived cells. In the earliest stages at which the cells were successfully karyotyped (after the establishment of mass cultures) all the cells were found to be diploid.

The chromosome constitution of early passage cultures was normal. However, with subsequent culture, partial deletions of one of the X chromosomes was evident, though this had no apparent effect on their differentiation. The extent of this deletion varies between HD lines, but the observation that the position of the break point is constant within a given line strongly suggests that, firstly, this phenomenon occurs early in their isolation and, secondly, that it does not arise by progressive deletion. It is interesting to note that the ESC stem cell line isolated by Martin (1981) is also reported as having a deletion of a single X chromosome. A more detailed analysis of the cytogenetic characteristics of these and other parthenogenetically-derived EK lines is currently being prepared (Robertson, Evans & Kaufman, 1983).

To date, no haploid mitoses have been observed in the established lines, and we can only speculate at which stage diploidization is occurring. We believe, from the morula studies indicated above, and from previous analyses of intact egg cylinders derived from haploid embryos (Kaufman, 1978*b*) that at least a proportion of the cells at explantation and shortly thereafter are still haploid.

While the success rate of establishing haploid-derived lines by the technique reported here is rather low, because of inevitable losses at each stage of the isolation procedure, attempts are being made to modify the explantation and cell isolation techniques in order to increase the chance of establishing both homozygous diploid as well as haploid pluripotential cell lines from this source. The HD lines reported here, which have been established from 1-pronuclear 'uniform' haploid embryos (Kaufman, 1981), clearly demonstrate that it is now possible to establish homozygous diploid pluripotent cell lineages of parthenogenetic origin which, at least initially, appear to be karyotypically normal, and capable of a full range of cellular differentiation.

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The early development and DNA content of activated human oocytes and parthenogenetic human embryos

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A total of 297 human oocytes that had failed to fertilize during in-vitro fertilization (IVF) cycles were exposed to the calcium ionophore A23187 to induce parthenogenetic activation. Of these oocytes, 192 (65%) activated, the majority (63%) exhibiting a single pronucleus and extruding a second polar body. The appearance of two pronuclei (18%) was generally associated with a failure to extrude the second polar body. Oocytes obtained from patients who were ≥35 years had a significantly reduced activation rate (53%). The timing of developmental events, such as extrusion of the second polar body, appearance and disappearance of pronuclei and the first two cleavage divisions, is broadly similar to that seen in fertilized oocytes. However, the developmental potential of human parthenogenetic embryos was reduced, as the majority of those allowed to continue in culture arrested between the 2-cell and 8-cell stages. Measurements of cellular DNA content using a computerized image analysis system showed that activated oocytes with one pronucleus had a DNA content compatible with a haploid number of chromosomes, while those with two pronuclei were diploid. The ability of parthenogenetically activated oocytes to replicate their DNA was also demonstrated.

Key words: DNA content/embryo research/human parthenogenesis/oocyte activation

Introduction

Knowledge of human embryology has undergone rapid expansion in recent years. Many of the advances achieved in the field of human early embryo development and their requirements for in-vitro culture have resulted from research utilizing embryos surplus to therapeutic requirements from in-vitro fertilization (IVF) programmes (Braude and Johnson, 1989). Such work has made significant contributions to the treatment of sub-fertility by assisted conception techniques and has also enabled the development of other uses of IVF, such as preimplantation diagnosis of genetic disease (Handyside *et al.*, 1989). However, the use of spare embryos for research has provoked intense ethical debate, which led in the UK to the passage of the Human Fertilization and Embryology Act (1990), which regulates their use. As cryopreservation of surplus embryos from IVF becomes increasingly available, the number of fertilized embryos donated for research is likely to decline. Although animal models such as the mouse have been used, there can be problems extrapolating from one species to another (Braude *et al.*, 1989; Pickering *et al.*, 1990). We have therefore investigated whether parthenogenetically activated, failed-fertilized human oocytes and their derivative cleavage stages might provide a suitable alternative to fertilized embryos for research.

There are a number of potential advantages to the use of parthenogenetically activated, failed-fertilized human oocytes in research. Oocytes which have failed to fertilize currently have no therapeutic use (Winston *et al.*, 1993a), and there is likely to be less ethical objection to the use of unfertilized oocytes rather than embryos for research. Activated oocytes may provide an alternative source of material with which to study the events occurring during human oocyte activation and early embryo development. Parthenogenetic embryos may also enable skills such as embryo biopsy to be acquired, and for the biochemical procedures needed for preimplantation diagnosis of genetic disease to be developed.

Parthenogenesis is the production of an 'embryo' (parthenote) from a female gamete without any contribution from the male gamete. Human oocytes normally arrest in their development at the diplotene stage of meiotic prophase I during fetal life. Further development does not occur until puberty, when a selected primary oocyte in each cycle responds to the luteinizing hormone (LH) surge and, with breakdown of the germinal vesicle, resumes meiosis I extruding the first polar body. At ovulation, the resulting secondary oocyte once again undergoes meiotic arrest but this time at metaphase II (Johnson and Everitt, 1989). Extrusion of the second polar body and completion of meiosis occurs only if triggered by an appropriate stimulus, usually fertilization, at which diploidy is restored.

Animal studies have demonstrated that stimuli other than fertilization can reinitiate meiosis at this point, resulting in parthenogenetic activation. Various physical and chemical stimuli have been used to induce activation experimentally in the mouse including exposure to electric shock (Tarkowski *et al.*, 1970), hyaluronidase (Graham, 1970), pronase (Kaufman, 1975), ethanol (Kaufman, 1982) and the calcium ionophore A2318 (Steinhardt *et al.*, 1974).

More recently, experimental parthenogenetic activation of human oocytes has been reported (Abramczuk and Lopata, 199(Johnson *et al.*, 1990; Winston *et al.*, 1991; Balakier and Caspe 1993). The method of parthenogenetic activation used for th study was exposure to the calcium ionophore A23187 which ha been shown to be a more effective activating agent for huma oocytes than ethanol (Winston *et al.*, 1991). A23187 probab induces activation by causing a rise in the level of intracellul

calcium (Vincent *et al.*, 1992). The aims of this study were to determine the activation rates of failed-fertilized human oocytes, the type of parthenogenetic activation produced, the timing of early stages of development of parthenogenetic embryos and the DNA content of activated oocytes and embryos.

Materials and methods

Source of oocytes for activation

Ethical approval to use spare oocytes donated by patients undergoing IVF or gamete intra-Fallopian transfer (GIFT) treatment at the Assisted Conception Research Unit, was obtained from the St Thomas's Hospital Ethical Committee and the Human Fertilization and Embryology Authority. Patients (n = 9) undergoing GIFT donated a total of 31 spare fresh oocytes, while those having IVF treatment (n = 68) donated a total of 297 oocytes which had been exposed to spermatozoa but remained unfertilized and had therefore been aged *in vitro*.

Ovulation induction for assisted conception procedures was achieved as described previously (Artley *et al.*, 1992), with human menopausal gonadotrophin (HMG; Pergonal, Serono, Welwyn Garden City, UK or Humegon, Organon, Cambridge, UK) following a long protocol of pituitary desensitization with a gonadotrophin-releasing hormone analogue (buserelin acetate; Suprefact, Hoechst, Hounslow, UK). Cycles were monitored using a combination of ultrasound and serum oestradiol estimation. Human chorionic gonadotrophin (HCG; Profasi, Serono) was administered when there were at least four follicles with a mean diameter of 17–19 mm, 34–36 h before egg retrieval. Oocytes were collected transvaginally under ultrasound guidance.

For patients undergoing GIFT treatment a maximum of three oocytes, with the prepared sperm sample, were transferred laparoscopically to a Fallopian tube. Donated spare fresh oocytes were not exposed to spermatozoa but were released from the cumulus mass by exposure to hyaluronidase (0.05%: Type II from ovine testes, Sigma Chemical Co., Poole, UK) in HEPES buffered Earle's balanced salt solution (HEBS; Braude, 1987), supplemented with 4 mg/ml bovine serum albumin (BSA fraction V; Sigma Chemical Co.) for 0.5–2.0 min. The oocytes were then washed in an excess of HEBS + BSA and left to rest for 1 h in HEBS + BSA under oil before being included in the activation protocol.

Oocytes obtained at egg collection from patients undergoing IVF cycles were cultured *in vitro* for 4–6 h before insemination. The culture medium used was Earle's balanced salt solution (EBSS; Braude, 1987), modified as described by Pickering *et al.* (1988), or modified T6 (Nasr-Esfahani *et al.*, 1990) supplemented with either 10% heat-inactivated patients' serum (HIS) or 10% human serum albumin (HSA) solution (Albuminar 20; Armour Pharmaceutical Co. Ltd, Eastbourne, UK) in 50 μl drops under oil in an atmosphere of 5% CO_2 in air.

Oocytes were examined for signs of fertilization ~18 h after insemination. Those without pronuclei and a second polar body were scored as unfertilized and were cultured for a further 4–8 h to ensure fertilization had not occurred before being included in the activation protocol.

Activation protocol

All oocytes were examined for the presence of a first polar body; those at the germinal vesicle stage were excluded from the protocol. The in-vitro aged oocytes were removed from their culture medium of EBSS or T6, supplemented with 10% HIS or HSA, and washed through HEBS + BSA. The oocytes were then left for 30 min in HEBS + BSA in a humidified dish without oil at 37°C (Winston *et al.*, 1991). This step had been shown previously to improve the activation rate, the likely explanation being that ionophore is highly lipophilic and may partition out to any lipids present in the culture medium, as would be the case when HIS supplementation is used.

The fresh or aged oocytes were then exposed to a 5 μM solution of calcium ionophore A23187 [Sigma Chemical Co.; stored as a 2 mg/ml stock solution in dimethyl sulphoxide (DMSO) at −70°C] for precisely 5 min. After three washes through EBSS + HSA, the oocytes were cultured at 37°C in 50 μl drops of the same medium under oil, in an atmosphere of 5% CO_2 in air. Oocytes were scored for signs of activation by examining for extrusion of a second polar body and the appearance of pronuclei.

Early development of activated oocytes

Activated oocytes and parthenogenetic embryos were examined regularly for changes in morphology, and the timings of cleavage events were recorded as hours post-activation (hpa). Activated oocytes were fixed at varying intervals after activation for analysis of their DNA content. Fixation was achieved using a 4% solution of formaldehyde in phosphate buffered saline (PBS; PBS tablets, Oxoid, Basingstoke, UK) for 30–60 min, followed by washing through an excess of PBS; the activated oocytes or parthenogenetic embryos were stored in drops of PBS under oil at 4°C. Control, in-vitro aged, failed-fertilized oocytes were fixed and stored in the same way.

Analysis of DNA content

Chromatin in the fixed material was stained with fluorochrome 4',6-diamidino-2-phenylindole.2HCl (DAPI; Sigma Chemical Co.) as a 5 μg/ml solution in PBS. The DNA content was estimated using a computerized image analysis system, Seescan (Seescan, Cambridge, UK). This system consists of a sensitive video camera linking the microscope with an ultraviolet (UV) light source to the PC-based Seescan computer. Material stained with DAPI was exposed to UV light and the resulting fluorescent image of the stained DNA 'captured', via the camera, onto the hard disc of the Seescan image analyser or to an optical disc drive. The resulting images could then be stored and recalled for later analysis.

The monochrome images of the recorded material were made up of pixels on the computer screen, each pixel discriminating between 256 grey scales from black (0) to white (256). The intensity of DAPI staining in the material exposed to UV light corresponded to the brightness of the pixels in the captured image.

An estimate of the nuclear DNA content was made from measurements of the intensity of staining in the cytoplasm of the cell and in the nucleus and the nuclear area, calculated by the computer after drawing around the relevant areas on the screen with a 'mouse'. The average intensity of the cell background was

subtracted from the average nuclear intensity to ensure that only the nuclear DNA was being measured. An integrated nuclear intensity value was calculated by taking into account both the corrected nuclear intensity and the nuclear area. Systematic timed studies of fluorescent intensity had shown there to be a precipitous drop in the level of stain in cells over the first hour after staining in fixed material, which plateaued to a steady state by 90 min after exposure to DAPI (data not shown; A.S.Taylor *et al.*, manuscript in preparation). All measurements were therefore carefully timed to be made 90 min after removal from DAPI. Unfertilized metaphase II oocytes were used as a control for a 2C DNA content and the values for activated oocytes and parthenogenetic embryos compared with these values.

Before measuring the DNA content of activated oocytes and parthenotes, the system was first validated using repeated measurements on fixed unfertilized human oocytes and fertilized mouse embryos that had been fixed at carefully timed stages of development (A.S.Taylor *et al.*, manuscript in preparation).

Results

Activation rates of fresh and in-vitro aged oocytes

A total of 297 failed-fertilized, in-vitro aged oocytes and 31 fresh oocytes were exposed to 5 μM calcium ionophore for 5 min. Of the aged unfertilized oocytes, 192 activated giving an activation rate for this group of 65%. The activation rate in the smaller group of 31 fresh oocytes obtained during the same period was 13/31 (42%), which is significantly lower than the rate for the in-vitro aged oocytes ($\chi^2 = 6.2$, $P = 0.013$).

Type of parthenogenetic activation

The majority of activated oocytes, 121/192 (63%), exhibited one pronucleus (1PN); 35/192 (18%) had two pronuclei (2PN); 10/192 (5%) had three pronuclei (3PN); and 6/192 (3%) had four or more pronuclear structures (≥4PN) (Figure 1). In 17/192 (9%) no pronuclei were seen (0PN) but second polar bodies were extruded and the oocytes later went on to cleave. These oocytes were generally examined for pronuclei later than usual and it is likely that the pronuclei were missed. Two of the oocytes that were fixed within 5 h of exposure to ionophore, in which pronuclei could not be seen, were found to be in anaphase on DAPI staining. The proportions of different types of activation were broadly similar in the small number ($n = 13$) of fresh activated oocytes.

Extrusion of second polar body by type of parthenogenetic activation

The presence or absence of a second polar body was recorded for a total of 150 activated oocytes that exhibited one, two or three or more pronuclei at activation. The vast majority of oocytes exhibiting one pronucleus at activation did extrude a second polar body (109/112 = 97%; Figure 2). Conversely, most of those with two pronuclei failed to extrude a second polar body (19/22 = 86%), suggesting that the appearance of two pronuclei in these oocytes was due to retention of the chromosomes destined for the second polar body. Approximately half (7/16) of the oocytes with three or more pronuclei extruded a second polar body.

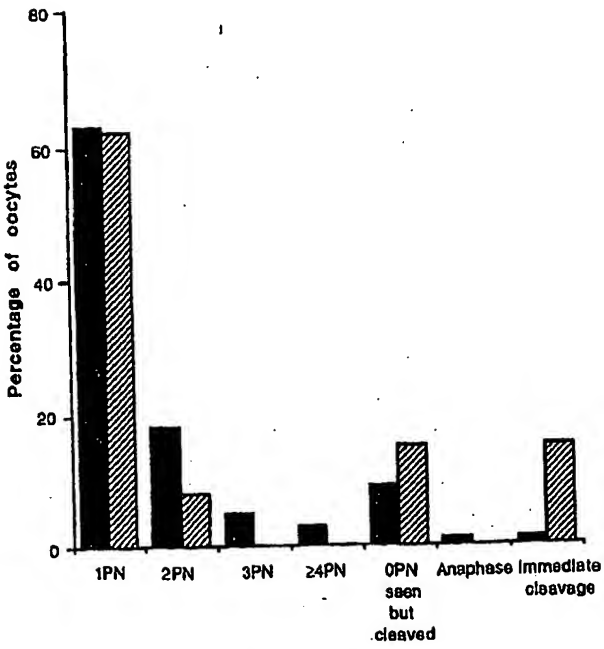

Fig. 1. Bar chart demonstrating the proportion of the different types of parthenogenetic activation (1PN; 2PN; 3PN; ≥4PN; 0PN seen but cleaved; anaphase on DAPI staining; and immediate cleavage) produced after exposure to calcium ionophore, A23187, in 192 activated, in-vitro aged, failed-fertilized human oocytes (solid bars) and 13 activated fresh oocytes (hatched bars). PN = pronucleus.

Factors that might affect the activation rate

Age

The patients were divided by age into three groups, 24–29, 30–34 and 35–39 years, to examine the effect of age on the activation rate of in-vitro aged oocytes. The activation rates were as follows: 56/74 (76%) in the youngest group, 74/107 (69%) in the 30–34 years age group, and 62/116 (53%) in the oldest group. The activation rates in the 24–29 and 30–34 years groups were not significantly different by the χ^2 test, but both groups had significantly higher activation rates than that for oocytes obtained from the oldest age group (35–39 years) (30–34 compared with 35–39, $\chi^2 = 5.8$, $P = 0.016$; 24–29 compared with 35–39, $\chi^2 = 9.5$, $P = 0.002$).

Semen

To examine whether the activation rate might be related to whether or not a male factor had contributed to the failure of fertilization, the oocytes were analysed in two groups: those obtained from cycles where the semen analysis was normal or abnormal. A normal semen analysis was defined as a count of $\geq 20 \times 10^6$ spermatozoa/ml, a progressive motility of ≥40% a normal morphology of ≥30% and a negative anti-sperm antibody screen. No significant difference was seen between the two groups; 85/127 (67%) oocytes activating in the normal semen group and 107/170 (63%) in the abnormal semen group.

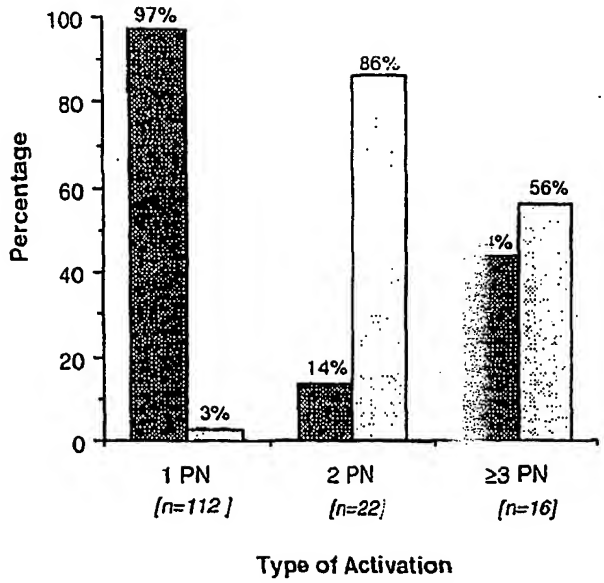

Fig. 2. Bar chart to demonstrate, by type of activation, the proportion of activated oocytes (n = 150) that extruded a second polar body (darker shading) and the proportion that failed to extrude a second polar body (lighter shading). PN = pronucleus.

Table I. Timing of different stages of development in human parthenogenetic embryos

Stage of development	Earliest seen (hours post-activation)	Latest seen (hours post-activation)
Extrusion of second polar body	5.5	7.5
Pronuclei observed	7.5	25
Cleavage		
2-cell	18	46
3-cell	25	44
4-cell	38	45
5-cell	41	69[a]
6-cell	46[a]	64[a]
7-cell	64[a]	65[a]

Apart from parthenotes that were fixed shortly after an observation ([a]), figures refer to activated oocytes or parthenote embryos that underwent at least one further stage of development after the recorded observation, and were not therefore arrested.

Complete failure of fertilization

Complete failure of fertilization in a cycle may be attributable to poor spermatozoa, poor oocyte quality or a combination of both. Although the activation rate of 57% (46/81) in oocytes from cycles with no fertilization was lower than that in cycles where there was some fertilization (68%; 146/216), this difference was not significant (χ^2 = 3.0, P = 0.08).

Pregnancy in the IVF cycle from which the oocytes were obtained

It might be thought that the overall quality of oocytes obtained from cycles which resulted in a pregnancy would be better than that of oocytes from unsuccessful cycles and therefore the former would be more susceptible to activation. However, the activation rate for oocytes from cycles resulting in pregnancy was 23/33 (70%), which was not significantly different from the rate for unsuccessful cycles (169/264; 64%).

Development of parthenogenetic embryos

Second polar body extrusion and pronuclei

Observations made on the timing of various stages of development suggested that these were broadly similar to those of normally fertilized embryos. The earliest and latest times that extrusion of the second polar body was seen were at 5.5 and 7.5 hours post-activation (hpa) respectively. The shortest time interval between extrusion of the second polar body and appearance of pronuclei was 2 h (Table I).

Pronuclei first appeared between 7.5 and 12.0 hpa and were present for 6–13 h in activated oocytes that went on to cleave (Figure 3A and B). The earliest time that pronuclei were seen to disappear was 14 hpa and the latest time that pronuclei were observed in parthenotes that did cleave was 25 hpa (Table I). If pronuclei persisted beyond this time it was associated with a failure to cleave.

Cleavage, fragmentation and embryonic arrest

The earliest and latest times that each of the cleavage stages was observed in non-arrested parthenote embryos are shown in Table I.

Of the 192 activated oocytes, 75 were fixed within the first 30 h post-exposure to ionophore for measurement of their DNA

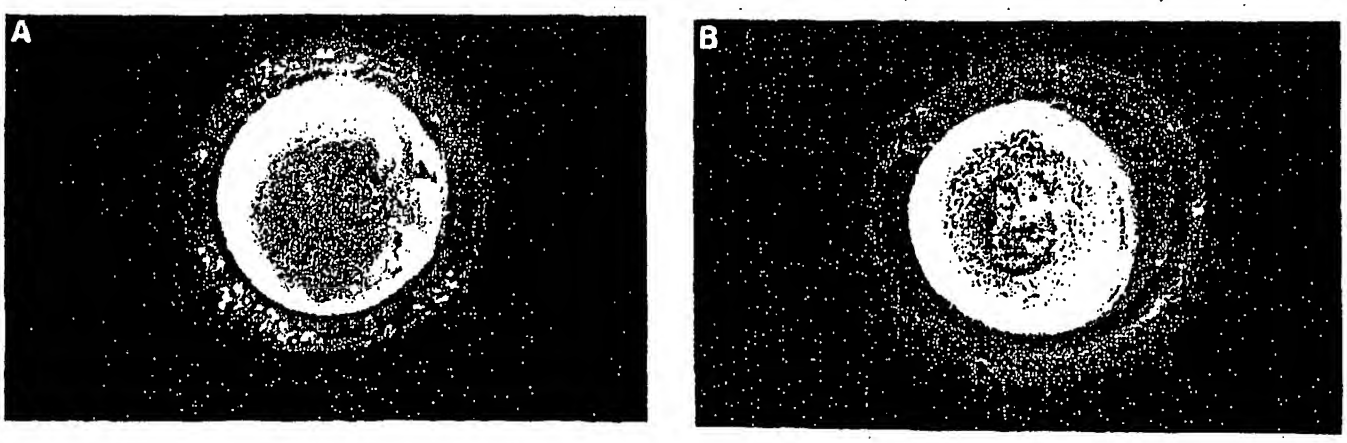

Fig. 3. (A) Parthenogenetically activated failed-fertilized human oocyte with one pronucleus and two polar bodies. (B) Parthenogenetically activated failed-fertilized human oocyte with two pronuclei after failing to extrude the second polar body.

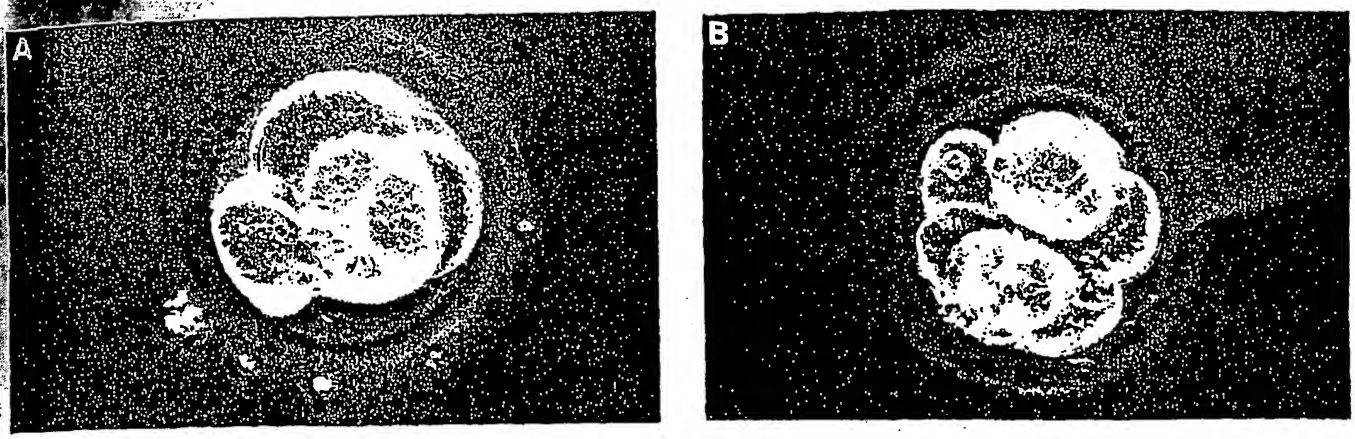

Fig. 4. (A) Cleavage stage 1 pronucleate human parthenote embryo with four cells. (B) Cleavage stage 1 pronucleate human parthenote embryo with seven cells.

content and 13 were used for [^{35}S]methionine-labelling, and therefore were excluded from the analysis of the cleavage potential of parthenote embryos. Of the remaining activated oocytes, 76% (79/104) underwent at least one round of cell cleavage (Figure 4A and B). Of the cleavage stage parthenote embryos, 57% (45/79) exhibited some degree of cellular fragmentation. As the majority of parthenotes (81/104 = 78%) were fixed in the first 72 h after activation for analysis of their DNA content, it is difficult to comment on the proportion exhibiting developmental arrest. In the small number of parthenote embryos (n = 23) allowed to continue in culture, all arrested by day 5 after activation, most (91%) by the 2- to 4-cell stages, and although none formed blastocysts, four parthenotes reached the 6- to 8-cell stages. There was no significant difference in the likelihood of cleavage or embryonic arrest when comparing 1PN with 2PN parthenogenetically activated oocytes.

DNA content of parthenogenetically activated oocytes

The numerical value obtained from the Seescan measurements of DNA content for both the control unfertilized metaphase II oocytes and the parthenogenetically activated oocytes within one experiment was divided by the mean obtained for the control oocytes and multiplied by two. This arithmetic manipulation resulted in the mean of the control group of oocytes, which should have a 2C DNA content, being standardized to a numerical value of 2, which facilitated direct comparison of DNA content with the activated oocytes (Figures 5–7).

DNA content of activated oocytes containing one or two pronuclei

Early after activation (5–8 hpa), oocytes with one pronucleus had a value for DNA content half of that for the metaphase II oocytes, i.e. 1C, while those with two pronuclei had the same DNA content as the controls, i.e. 2C (Figure 5). This further verified accuracy of the image analysis system and confirmed the expectation that 1PN human parthenotes are haploid while those with two pronuclei are diploid.

DNA replication of parthenogenetically activated oocytes in the first 24 h post-activation

Activated oocytes were fixed at varying times within the first 24 h after activation to estimate their DNA content (Figure 6).

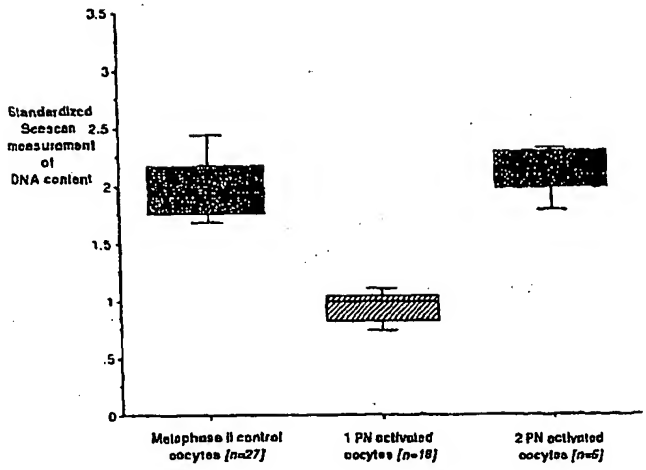

Fig. 5. Box plots of standardized Seescan measurements of DNA content for activated oocytes at 5–8 h post-activation (hpa) with one or two pronuclei (PN), using metaphase II oocytes as a control for a 2C DNA content. The upper and lower borders of the boxes represent the 75th and 25th centile values respectively, the bar horizontally across the box is the median and the 'whiskers' are the 10th and 90th centiles.

The DNA content halved from 2C to 1C between 5 and 8 hpa, but by 15 hpa the DNA content of activated oocytes had started to increase. Between 15 and 24 hpa the DNA content gradually increased further, resulting in restoration of an intensity equivalent to a 2C DNA content by 24 hpa.

DNA content of 1PN cleavage stage embryos

Measurements made on 1PN activated oocytes fixed within 1–2 h of cleavage to 2-cell parthenote embryos showed that each cell has an estimated DNA content equivalent to half the control oocytes and the activated oocytes at 24 hpa, i.e. a 1C DNA content (Figure 7).

Discussion

Other studies have reported human oocytes to be particularly resistant to parthenogenetic activation using agents such as ethano

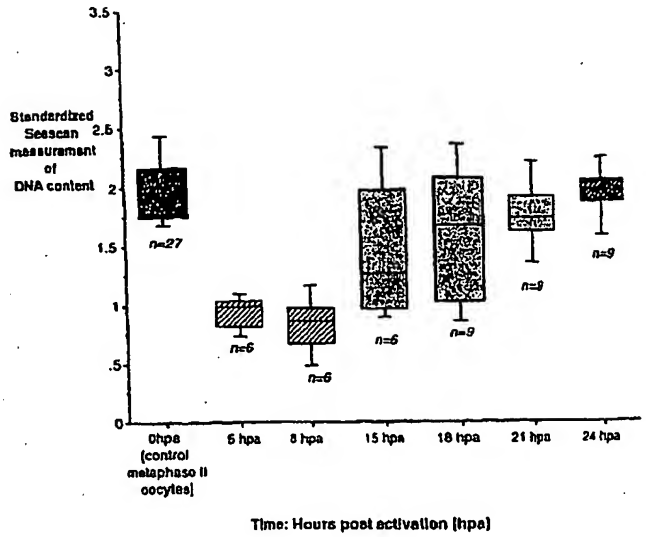

Fig. 6. Box plots (as described for Figure 5) of standardized Seescan measurements of pronuclear DNA content in parthenogenetically activated human oocytes, fixed between 5 and 24 h post-activation (hpa).

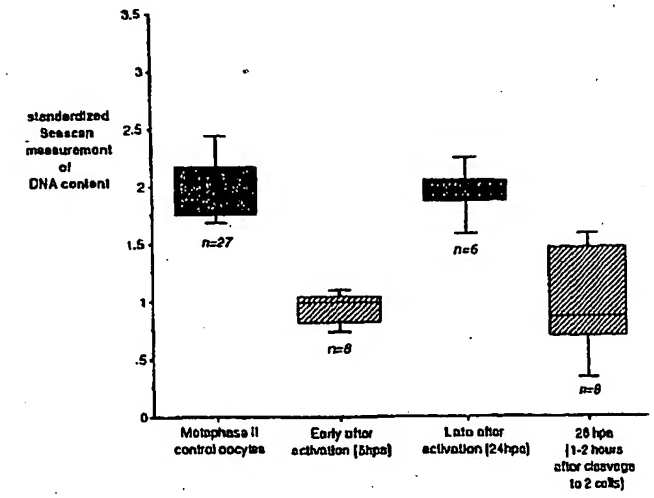

Fig. 7. Box plots (as described for Figure 5) of standardized Seescan measurements of DNA content in parthenogenetically activated human oocytes early [5 h post-activation (hpa)], late (24 hpa) after activation and within 2 h of the first mitotic cleavage.

(Abramczuk and Lopata, 1990; Winston *et al.*, 1991; Balakier and Casper, 1993). Using the calcium ionophore, A23187, we have been able to activate consistently 65% of failed-fertilized, in-vitro aged, human oocytes. Although this activation rate is not as high as that achieved with ionophore in aged mouse oocytes (Kaufman, 1983; Vincent *et al.*, 1992), it is higher than that reported previously for ionophore-induced activation of human oocytes (Balakier and Casper, 1993), where only 16% of oocytes were activated. The protein synthesis inhibitor puromycin may be a more effective activator of failed-fertilized human oocytes, with reported activation rates of ~90% (Balakier and Casper, 1993; De Sutter *et al.*, 1993); however, the potential for further development and cleavage may be lower.

The activation protocol followed in this study was as described by Winston *et al.* (1991), and included a 30 min period of washing through medium containing no human serum or serum-derived proteins which could contain traces of lipids or lipoproteins. Calcium ionophore is highly lipophilic and its activity may be reduced by small traces of lipids or lipoproteins within the culture medium. Winston *et al.* (1991) had shown that optimal exposure to ionophore was at a 5 μM concentration for 5 min, and that longer exposures or higher concentrations were more likely to be associated with oocyte damage.

Although the number of fresh oocytes in this study was small in comparison with the in-vitro aged oocytes, the activation rate was nevertheless significantly higher in the aged oocytes. This is compatible with Kaufman's finding of an increased activation rate in mouse oocytes with increasing time after ovulation (Kaufman, 1983), and may reflect a decline with time in the cytostatic factors holding oocytes at the metaphase II block. Interestingly, the high activation rate of human oocytes (90%) reported by De Sutter *et al.* (1993) was observed in oocytes that had been aged *in vitro* for 2–3 days following oocyte retrieval before exposure to the activating stimulus.

It has been suggested that the appearance of spontaneously activated oocytes in an IVF cycle is associated with an increased chance of pregnancy in that cycle (Jackson *et al.*, 1992). In this study the induced activation rate was no different for oocytes obtained from successful cycles in which a pregnancy ensued, when compared with the activation rate in oocytes from unsuccessful cycles. It was interesting to observe, however, that oocytes obtained from patients >34 years old were significantly less likely to activate. This finding would be consistent with the suggestion that there may be a reduction in the quality of oocytes obtained from older patients undergoing IVF, and could in part explain the lower fertilization rates and success rates reported for these patients.

Oocytes that have failed to fertilize in cycles where there was a normal semen analysis might be more likely to be abnormal or to have contributed in some way to the failure of fertilization, and thus be less likely to activate. However, it was found that the activation rate for these oocytes was the same as for those from cycles where there might be a male factor to account for the failure to fertilize. Likewise, there was no significant difference in activation rate in oocytes obtained from cycles in which there had been no fertilization at all, and those from cycles where some of the oocytes did fertilize.

The technique of using a computerized image analysis system (Seescan) for measuring the DNA content of Feulgen-stained megakaryoctyes has been reported previously (Woods and Trowbridge, 1990). The fluorescent DNA stain, DAPI, has also been used to quantitatively assess cellular DNA content photocytometrically (Coleman *et al.*, 1981; Winston *et al.*, 1991, 1993b). The use of a computerized image analysis system to measure the DNA content of DAPI-stained cells has not been reported previously. The technique is simple and has a number of advantages. Firstly, it can be used on both live and fixed material because the image enhancement facility within the system

allows the concentration of stain used and the length of exposure to UV light required to capture an image to be extremely small. Secondly, once captured, the images can be analysed at leisure at a later time, of particular benefit where live material is concerned. The ability to accurately delineate the nuclear area is also an advantage, especially where this shape is not regular or geometric, such as two overlapping pronuclei. It is possible with this technique to distinguish the gross order of DNA content within a cell, e.g. 1C or 2C, but the technique would not be sensitive enough to distinguish more subtle differences in numbers of copies of individual chromosomes. It was possible to demonstrate a clear difference between 1PN and 2PN activated oocytes; those with 1PN having half the DNA compared with those with 2PN or control unfertilized metaphase II oocytes. Unfertilized human metaphase II oocytes were an easily available and appropriate control for a 2C DNA content.

The majority of activated oocytes exhibited one pronucleus, extruded a second polar body and were confirmed on image analysis after DAPI staining to have a DNA content equivalent to 1C, compatible with a haploid number of chromosomes. In contrast, the smaller proportion with 2PN generally failed to extrude a second polar body and DNA measurements were compatible with a diploid chromosome complement.

The measurements of DNA content over the first 24 h after activation indicate that DNA replication can occur in parthenogenetically activated human oocytes, even in the absence of the paternal genome. Replication begins at 8 hpa and is complete by 24 hpa. DNA replication during the S phase of the first cell cycle of fertilized human zygotes has been reported to start at 9–10 h after insemination and be completed 3–5 h later (Balakier *et al.*, 1993). We have shown here that the DNA content of individual cells in 2-cell parthenote embryos had reduced back to a 1C level immediately after cleavage, indicating that cleavage is possible in human parthenotes with a haploid chromosome complement.

The timing of the appearance and disappearance of pronuclei was variable but generally was similar to that seen in normally fertilized oocytes. Where activated oocytes were observed serially every hour after activation, it was noted that the earliest time that pronuclei were no longer visible was at 14 hpa. This may explain the fact that for some oocytes examined for pronuclei later than usual, at 18–20 h after exposure to ionophore, no pronuclei were observed but the oocytes later cleaved.

Three quarters (76%) of the activated oocytes went through at least one round of cell division, indicating that cleavage is possible in the absence of the paternal genome. At the light microscope level, parthenogenetic embryos appear morphologically similar to early cleavage stage fertilized embryos (Figure 4A and B). It is difficult to draw any firm conclusions about the developmental potential of human parthenote embryos, as the majority of activated oocytes in these experiments were fixed for analysis of their DNA content. It is possible, however, that the potential for cleavage is limited, as the majority (91%) of the small number allowed to continue in culture had arrested by the 4-cell stage, and cleavage to seven cells was the furthest stage any parthenote embryo reached by day 5 after activation. This is in contrast to the development of parthenogenetic embryos in other mammalian species. Studies of mouse parthenotes have shown that they are capable of developing as far as post-implantation stages, although development then arrests probably due to poor placental development (Tarkowski *et al.*, 1970; Graham, 1974; Kaufman, 1983). Parthenogenetic or experimentally generated digynic mouse embryos exhibit a pattern of preimplantation development that is indistinguishable from fertilized embryos as far as the blastocyst stage (Howlett *et al.*, 1990). However, after implantation these embryos fail to develop normally and exhibit poor trophoblast development (Surani and Barton, 1983). In contrast, androgenetic embryos are much less likely to develop normally before implantation but if they do survive to implant, trophoblast proliferation can be extensive and is associated with poor embryonic development (Barton *et al.*, 1984), a situation that is analogous to hydatidiform moles in humans (Szulman, 1988). Thus, both maternal and paternal genetic contributions are required to achieve normal development in the mouse. Such genomic imprinting may also preclude normal development of human parthenogenetic embryos, although the stage at which arrest may occur is yet to be defined.

Poor cleavage in human parthenotes could also be due to a lack of cytoplasmic factors that are normally gained at fertilization together with the male genome. It has been suggested that human centrioles are paternally derived from the mid-piece of the spermatozoon (Sathananthan *et al.*, 1991). If these were essential for normal cleavage, their absence after parthenogenetic activation could contribute to abnormal development. However, ultrastructural examination of fertilized mouse oocytes for centrioles has shown that although a structure similar to the sperm centriole was found with the remnants of the sperm axoneme, centrioles were not observed at either pole of the first mitotic spindle, thus questioning the function of centrioles in mammalian cells (Schatten *et al.*, 1985).

Although centrosomes have been demonstrated to be paternally derived in species such as sea urchins, in mice the centrosomes may be maternally inherited, again suggesting that the inheritance of centrosomes in mammals may be different (Schatten *et al.*, 1989). It is therefore unclear whether all that is needed for preimplantation development is already present in human oocytes as it appears to be in the mouse, and if not, exactly what paternally derived factors are critical.

While the reason for the developmental arrest in human parthenogenetic embryos is uncertain, it does not seem to be related to whether the embryos were 1PN (1C, presumed haploid) or 2PN (2C, presumed diploid).

Another possible explanation for cleavage arrest in parthenotes is that they fail to activate their embryonic genome, although failure to activate the genome has only been demonstrated in half the normally fertilized embryos which undergo cleavage arrest *in vitro* (Artley *et al.*, 1992). Further work is currently in progress to determine whether these embryos exhibit the characteristic patterns of protein synthesis that are seen in fertilized embryos before and after embryonic genome activation (Braude *et al.*, 1988).

Alternatively, parthenote embryos may have different culture requirements to normally fertilized embryos. The culture media used were the same as those for fertilized embryos in the clinical

IVF programme at the time and the poor development beyond the 4-cell stage may reflect sub-optimal culture conditions for cleavage of parthenote embryos.

In conclusion, the calcium ionophore A23187 is an effective activating agent for failed-fertilized human oocytes. Three quarters of activated oocytes undergo cell cleavage, although the developmental potential of human parthenogenetic embryos may be limited. Further work is needed to clarify optimal culture conditions for activation and subsequent development, as well as to determine the reason for the developmental arrest and whether this can be overcome. If it proves possible to demonstrate normal biochemical events such as protein synthesis, and if cleavage is more reliable, human parthenogenetic embryos might provide a useful alternative to human embryos for research.

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